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# *In vivo gene therapy of malignant tumours with heat shock protein-65 gene*

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*We have previously shown that ex vivo insertion of a gene encoding the mycobacterial heat shock protein-65 into tumour cells results in their inability to form tumours in mice. We report regression of highly malignant reticulum cell sarcomas (J774) after liposome-mediated gene transfer in vivo. Heat shock gene transfer resulted in tumour regression both in immunocompetent and immunodeficient SCID mice. Complete tumour eradication, however, was detected only in immunocompetent animals, confirming the role of T cells in tumour rejection. Treatment of tumour bearing mice with the heat shock gene–liposome complex resulted in the production of antibodies against the tumour cells, indicating an increase in the antigenicity of the tumour after gene transfer. These results suggest that the heat shock protein-65 gene could provide a novel approach for the treatment of established tumours.*

**Keywords:** *heat shock protein; cancer; gene therapy; Mycobacterium*

## *Introduction*

Heat shock proteins (hsp) are induced by various forms of cellular stress to protect cells from environmental damage.[1,2] Many members of the hsp family are also essential for cellular functions under physiologic conditions.[3,4] Involvement in protein folding and transport led to designation of hsps as molecular chaperones.[5] Hsps can chaperone small antigenic peptides to MHC molecules for efficient antigen presentation.[6] It has been reported that mice immunised with tumour-derived hsp fractions associated with tumour-specific small peptides, develop antitumour protection specific for the parent tumour cells.[7]

In addition to functioning as molecular chaperones, bacterial hsps are among the most potent antigenic stimuli for the mammalian immune system. We have previously shown, that tumour cells expressing a mycobacterial hsp gene after *in vitro* gene transfer lose their ability to form tumours and induce a preventive antitumour immune response.[8,9] In this study we show the effectiveness of hsp65 gene transfer *in vivo* in the treatment of existing tumours.

## *Results*

### *Increased antitumour protection after hsp gene transfer in vivo*

Immunocompetent Balb/c and T cell-deficient SCID mice were injected with $2 \times 10^6$ J774 cells into the peritoneal cavity. Four days later they received an intraperitoneal (i.p.) injection of 100 μg hsp65 DNA complexed with liposome (DOTAP). The treatment was repeated twice weekly for 2 weeks. Tumour progression was compared to untreated and vector DNA–liposome-treated controls. Untreated and vector DNA–liposome-treated mice developed highly malignant lymphoreticular neoplasms composed of histiocytic cells growing in the peritoneal cavity and in most abdominal organs. Control mice receiving vector DNA–liposome injections showed a small but significant decrease in tumour size ($P < 0.05$). Hsp65–liposome gene therapy resulted in a large and highly significant ($P < 0.0001$) reduction in tumour size in immunocompetent animals by day 18 compared with both the 20 untreated and the 10 vector-treated controls (Figure 1a). After hsp gene therapy palpable tumours were found in three out of 20 immunocompetent Balb/c mice. SCID mice receiving hsp65 gene therapy also developed smaller tumours ($P < 0.01$) than the untreated and vector–liposome-treated controls (Figure 1b), but in 80% of the hsp-treated mice palpable tumours were detectable at autopsy.

Flow cytometric DNA analysis of peritoneal samples obtained from tumour bearing Balb/c mice confirmed the presence of highly proliferative tumour cells (Figure 2a). Hsp65–liposome treatment initiated on day 4 (as described for Figure 1) resulted in a significant decrease in the number of cells in G2/S phase (17.3 ± 5.4%) (Figure 2b) compared with 10 vector–liposome-treated controls (74.0 ± 6.3%) ($P < 0.001$). DNA histograms obtained from peritoneal samples of hsp65 gene-treated SCID mice showed a decrease in S/G2 phase cells to 64.4 ± 12.3% (Figure 2d), compared with 85.6 ± 2.3% in vector–liposome-treated controls (Figure 2c). This difference was not statistically significant ($P < 0.1$).

PCR analysis confirmed the expression of hsp65 mRNA in peritoneal tumour samples of SCID mice receiving hsp–liposome gene therapy. DNase-treated samples obtained from hsp–gene-treated SCID mice contained an hsp65-specific mRNA band not seen in

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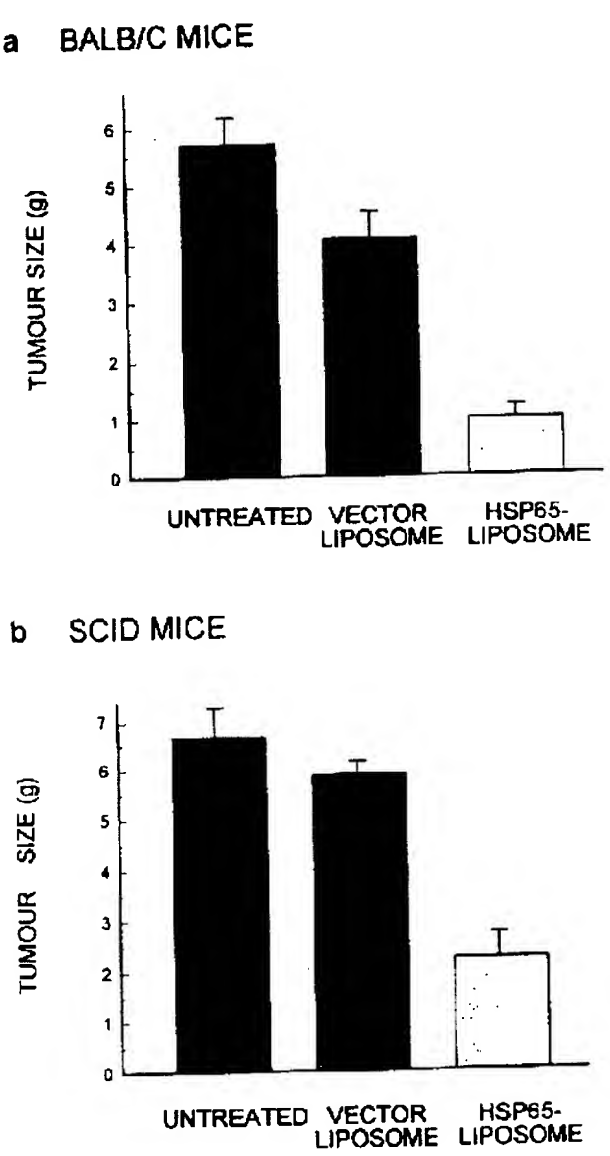



*Figure 1 Effect of in vivo hsp65 gene therapy in immunocompetent Balb/c mice (a) and immunodeficient SCID mice (b). Fifty Balb/c mice and 15 SCID mice received 2 × 10⁶ J774 tumor cells i.p. Four days later 20 Balb/c mice and five SCID mice were injected with 100 μg hsp65 plasmid DNA complexed with 400 μg liposome (DOTAP) in 1 ml physiologic saline i.p. Vector-treated controls (10 Balb/c and five SCID mice) received 100 μg plasmid DNA complexed with 400 μg DOTAP. The treatment was repeated twice weekly for 2 weeks. Tumour sizes were determined 3 days after the last treatment as described in Materials and methods (mean ± s.e.).*

untreated and vector–liposome-treated animals (Figure 3). The positive control, β-actin was detected in all samples. Peritoneal cells and abdominal organs (spleen, liver and kidney) obtained from immunocompetent Balb/c mice after hsp–liposome gene therapy did not express hsp65 mRNA (data not shown).

To assess the effect of hsp65 gene transfer on advanced tumours, experiments were carried out in Balb/c mice 2 weeks after tumour implantation. Mice were injected with $2 \times 10^6$ cells into the peritoneum. Fourteen days later they received the first injection of hsp65–liposome, repeated at 2 day intervals on four occasions. On day 21 autopsy was performed to determine if tumours were present and measure tumour size. In seven out of 10 hsp–liposome-treated mice no palpable tumour was found at autopsy and there was a significant decrease in tumour size compared with the 10 untreated controls ($P < 0.001$) (Figure 4). J774 reticulum sarcoma is a tumour with aggressive *in vivo* growth characteristics ($2 \times 10^6$ tumour cells injected i.p. cause death by day 22–26). Rapid regression of advanced tumours (Figure 4) suggests that hsp65 gene transfer can be an effective treatment in late cancer. It proved to be essential to complex the hsp65 DNA to liposomes, since treatment of tumour bearing mice with peritoneal injections of naked DNA or pre-immunisation of mice with intramuscular injections of naked DNA have not resulted in significant antitumour protection (data not shown).

### *Production of tumour-specific antibodies after in vivo hsp gene therapy*

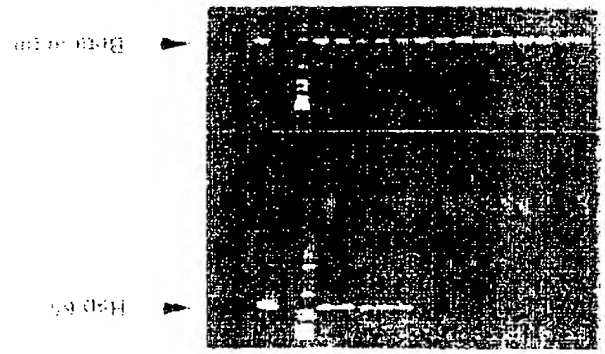
Balb/c mice injected with J774 tumour cells alone produced no antibodies against the tumour (Figure 5). Treatment of mice with vector–liposome complexes had no effect on antibody production. Intraperitoneal injections of the hsp65 gene complexed with liposomes into tumour bearing mice (as described for Figure 1) resulted in production of tumour-specific antibodies. Various dilutions of sera from hsp gene-treated animals tested by ELISA assay using the lysate of nonmodified J774 cells contained J774-specific antibodies but did not react with lysates from other tumours (Wehi-164 and Pu-518) (data not shown). These results confirm increased recognition of tumour-specific structures after hsp gene transfer.

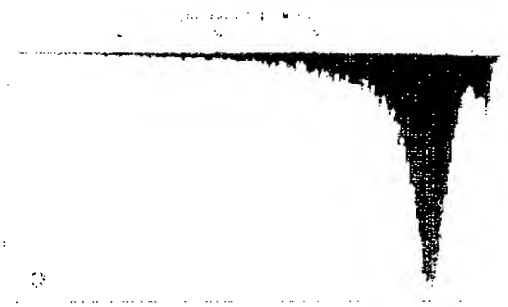
## *Discussion*

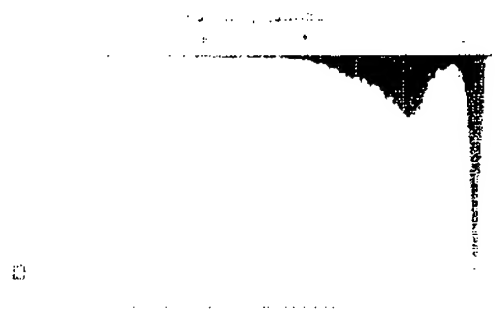
Our results demonstrate regression of established reticulum cell sarcomas after *in vivo* gene therapy with the mycobacterial hsp65 gene. Complete tumour rejection was achieved only in Balb/c mice with intact T cell-mediated immunity. On the other hand, reduction in tumour size observed in hsp gene-treated SCID mice suggests that T cell-independent mechanisms such as NK cells or macrophages or changes in the cell cycle regulation (Lukacs, unpublished) may also contribute to tumour reduction.

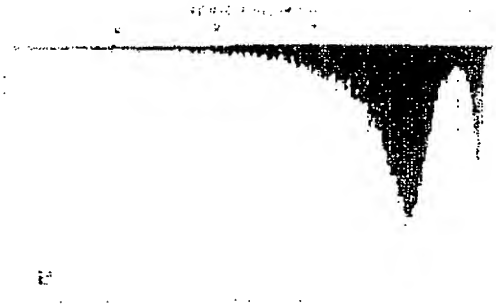
The inserted mycobacterial hsp65 gene may elicit an immune response by several mechanisms. Although hsps are highly conserved proteins, species-specific sequences are recognised as foreign epitopes by the immune system.[10,11] The mycobacterial hsp65 is capable of inducing strong cellular and antibody responses.[12] Peptide vaccines containing mycobacterial hsps complexed with weakly antigenic peptide epitopes from pathogens have been shown to induce strong immune responses specific for the peptides.[13,14]

The highly immunogenic nature of the foreign 65 kDa hsp molecule may similarly provide greatly enhanced, associated-recognition of tumour-associated antigen(s). In preliminary experiments, J774 cells transduced with the mycobacterial hsp65 gene have been shown to be more effective as antigen presenting cells than unmodified or vector-transduced control cells (B Stockinger, unpublished), suggesting that more efficient presentation









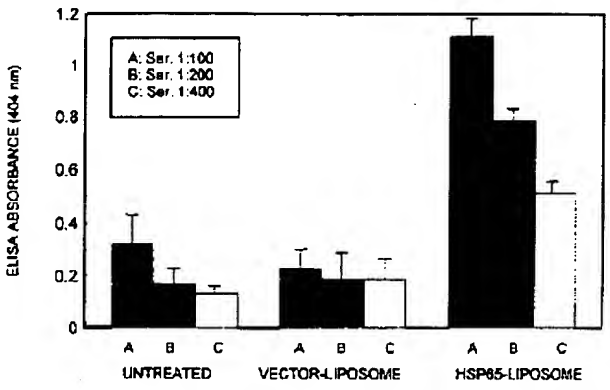



*Figure 5 Detection of tumour-specific antibodies in the sera of mice after in vivo hsp65 gene therapy. Serum was collected from tumour-bearing Balbic mice treated with hsp65 gene–liposomes, vector–liposomes or untreated controls (administration of treatment was as described for Figure 1). Serum samples in each group were pooled and various dilutions were tested on lysates of nonmodified J774 cells in an ELISA assay.*

of tumour associated antigens could play a role in the induction of antitumour immune response.

Studies by Srivastava and his colleagues[6,7,15] have demonstrated that mammalian hsps isolated from tumour cells elicit protective immunity. Identical hsp fractions from normal tissues are not effective and tumour-derived hsps lose their protective effect after elution of small tumour-specific peptides[16] confirming that hsps function as chaperones for tumour-derived peptides. In our studies, the presence of tumour-specific cytotoxic T cells elicited by hsp-transduced J774 cells *in vivo* (capable of lysing both hsp-transduced and nonmodified tumour cells)[8,9] and production of J774-specific antibodies in mice receiving hsp65 gene transfer (Figure 5) provide experimental evidence for increased recognition of tumour antigens *in vivo*. Hsp65 has been detected in the supernatant of hsp-transfected cells,[17] it could also effectively transport (and protect from degradation) tumour-derived peptides to antigen presenting host cells eliciting immune response through both the MHC class I[18] and class II[19] dependent pathways. Since the majority of human tumours express low levels of MHC molecules, making direct presentation of their antigens to T cells ineffective, this mechanism may be crucial for the induction of an effective antitumour immune response in humans.

## Materials and methods

### Cells and cell cultures

J774GS cells (J774) were maintained in DME with 10% fetal calf serum, 100 μg/ml streptomycin, 100 μg/ml penicillin, 1 mM L-glutamin and 10 mM Hepes.

### Inoculation of mice with tumour cells

Groups of 6 to 12-week-old female Balb/c and SCID mice were obtained from breeding colonies at the National Institute for Medical Research. For the production of tumours mice were inoculated intraperitoneally with $2 \times 10^6$ J774 tumour cells taken from subconfluent cell cultures.

### Assessment of tumour size

Since tumours grew in most abdominal organs, resulting in an inseparable aggregation of primary tumours and abdominal organs, tumour size was calculated by weighing the primary tumour and abdominal organs of the test mice, and subtracting the average weight of abdominal organs obtained from five age-matched normal controls.

### In vivo gene transfer

At various time-points after tumour induction, mice received four i.p. injections in 2 weeks, each containing 100 μg plasmid DNA complexed with 400 μg liposome (DOTAP; Boehringer Mannheim, Lewes, East Sussex, UK) at room temperature for 15 min before injection. Animals receiving the liposome-complexed plasmid DNA encoding the mycobacterial heat shock protein-65 (pZIPML65)[17] were compared with untreated controls and mice injected with liposome-complexed control plasmid (pZIPNeoSVX).

### DNA histograms

Transfected and control cell lines, and cells obtained from the peritoneal cavity of mice were incubated with DNA specific dyes and assessed by flow cytometry[20] (FACScan; Becton Dickinson UK, Oxford, UK). Peritoneal cell samples were first fixed with 1% paraformaldehyde in PBS, then incubated with 50 μg/ml propidium iodide (Sigma Chemical, Poole, UK) for 30 min on ice. DNA histograms were obtained for 10000 cells in each sample.

### mRNA

Total RNA was isolated from $2 \times 10^6$ peritoneal cell samples and approximately $2 \times 3$ mm sections of various organs using guanidium thiocyanate–phenol–chloroform extraction and alcohol precipitation.

After DNase treatment reverse transcription was carried out as previously described.[21] PCR amplification was performed using oligonucleotide mycobacterial hsp65-specific primer pairs (5′-TTGAGCAGGTCCTCCTAC TCA-3′ and 5′-ATGGCCAAGACAATTGCCTAC-3′) synthesised at the National Institute for Medical Research. The PCR mixture containing 1.25 μl of each primer at 20 μM was subjected to the following PCR conditions on an Omnigene (Hybaid, Middlesex, UK) TR3 SM2 thermocycler: 35 cycles of 96°C/45 s; 60°C/45 s; 72°C/90 s, followed by an extension cycle of 72°C for 5 min. The β-actin primers were used as a control for both reverse transcription and the PCR reaction itself. After amplification, 10 μl of reaction product and 2 μl DNA loading buffer were electrophoresed in 1.5% agarose containing ethidium bromide (0.5 μg/ml; Sigma Chemical) in 1 × Tris-acetate EDTA buffer; molecular weight (mw) standards (kb ladder, BRL) were included. Bands were visualised using a UV transilluminator and 65 kDa hsp-specific bands were identified by anticipated mw. Hsp65 plasmid DNA (0.1 μg/ml) was PCR amplified after reverse transcription and included as a positive control.

### Detection of J774-specific antibodies

Sera were collected from tumour bearing mice after *in vivo* gene transfer as described for Figure 1. J774 ($10^5$) cells were sonicated on ice in 1 ml of 0.25 M carbonate buffer pH 9.6. Cell lysates (100 μl per well) were incubated in Maxisorp F96 microtiter plates (Nunc, Roskilde, Denmark) at 4°C overnight to allow absorption of anti-

genic structures to the plate. Wells were incubated with 200 µl of 1% BSA for 4 h to minimise nonspecific binding. Serum samples diluted in blocking buffer (1% BSA in PBS) were added to the wells and incubated for 2 h at 37°C. J774-specific antibodies were detected by anti-mouse IgG-alkaline phosphatase conjugate and 104 phosphatase substrate (Sigma Chemical) by an ELISA plate reader.

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